Cubebin PLGA microparticles preparation and characterization

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Introduction

Nowadays the vegetal origin products represent approximately half of the drugs in used and the interesting in this research area has been creased in view of the large number of compound biologically active that can be isolated of plants (Montanari et al., 2001). The Cubebin belong to the dibenzylbutyrolactone lignan class of compounds, which is among the natural products of interest, since many of its compounds display a broad range of biological activities with therapeutic potential (Piccinelli et al., 2005; de Souza et al, 2004; da Silva et al., 2005).

As previously demonstrated oral administration of Cubebin displays significant anti-inflammatory and analgesic activities (Bastos et al., 2001). However, despite of the significant trypanocidal activity demonstrate for the *in vitro* assay, Cubebin usefulness for Chagas disease is limited by the lower activity *in vivo*, when administrated intraperitoneally (Bastos et al., 1999).

The use of biodegradable polymers, as PLGA, for the controlled release of therapeutic agents is now well established. The microparticles were able to sustain the release of the drug for a considerable period of time reducing the required frequency of administration increasing patient compliance, avoid plasmatic fluctuations, decrease side effects and facilitate dosage administration (Hans et al., 2002).

In the present work, Cubebin loaded PLGA microparticles was prepared and characterized by physical-chemical methods. *In vitro* release studies should be made to evaluate the drug availability. Considering the need to determine the amount of Cubebin in pharmaceutical formulations a high performance liquid chromatography (HPLC) method was developed and validated.

Materials and methods

(-)-Cubebin was isolated from *Piper cubeba* L. according to Bastos et al. (1996). Its chemical structure was confirmed by ^{1}H NMR and IR data, as previously reported (Souza et al., 2004). The preparation of PLGA microparticles was performed by the classical emulsion solvent-evaporation method (Birnbaum et al., 2000; Niwa et al., 1994). The shape and surface topography of the dry microparticles were observed under the scanning electron microscope (Leica Model Stereoscan 440). The size and size distribution of microparticles were evaluated by a Light Scattering particle size analyzer (LS^{TM} 13 320, Beckman Coulter).

The chromatographic analyses was performed on C_{18} reversed-phase column shim-pack (250x4mm, particles of 5µm) and a C_{18} pre-column shim-pack (4x4mm, particles of 5µm), at controlled room temperature (25°C). A HPLC method was developed and then a (60:40, v/v) acetonitrile:water was used as mobile phase, at flow rate of 1.0mL/min. The HPLC method was validated for assay of Cubebin according with both norms of ICH topics Q2A e Q2b (1996) and of ANVISA Resolution - RE nº 899 (2003). Precision was calculated from the coefficient of variation (CV%) of the standard curve and linearity was assessed from the linear regression. The limit of quantification was defined. The repeatability and accuracy were assessed.

For determinate the percentage of drug encapsulated a known amount of weighed lyophilized microparticles (2mg) was dissolved in 4mL of dichloromethane, shacked vigorously during 1 min and sonicated for 1 min. Then, the polymer was precipitated with the addition of 19mL of ethanol. The suspension was mixed, centrifuged and filtered through a membrane filter and analyzed by HPLC as described below. The percentage of drug encapsulated in the PLGA microparticles was represented by Equation: Encapsulation efficiency (%) = total amount of drug determinate in microparticles × 100 /total amount of drug theoretically associated with microparticles. The selectivity of assay was determined by placebo analysis. Placebos of microparticles formulations containing all the normal ingredients except Cubebin were prepared for this study.

Results and Discussion

Administration of drugs using biodegradable PLGA polymers has generated great interest due to its excellent biocompatibility and biodegradability. Cubebin PLGA microparticles were prepared with success by the emulsion evaporation method. The microparticles formed presented narrow distribution size and a mean diameter of of 3,798µm ± 1,945µm (Figure 2).

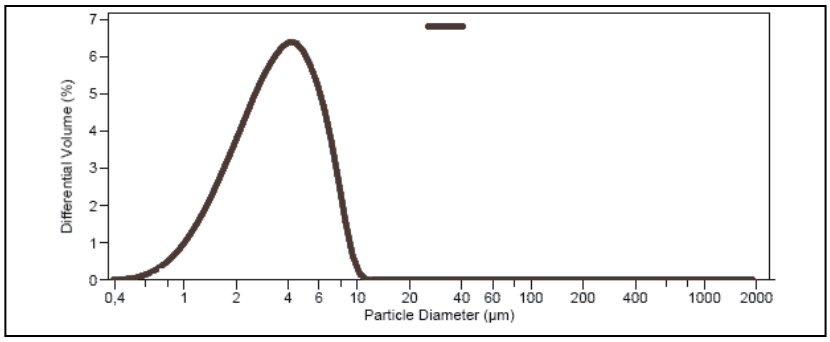

Figure 2. Size distribution of Cubebin loaded PLGA microparticles.

Furthermore, scanning electron micrographs of PLGA microparticles obtained showed that it presented smooth and spherical surface (Figure 3). Satisfactory yield and encapsulation efficiency of Cubebin were obtained (80%). In according others authors showed that the conventional oil-in-water (o/w) emulsion-solvent evaporation method used are adequate to encapsulated lipid-soluble drugs, like Cubebin (Cegnar et al., 2005).

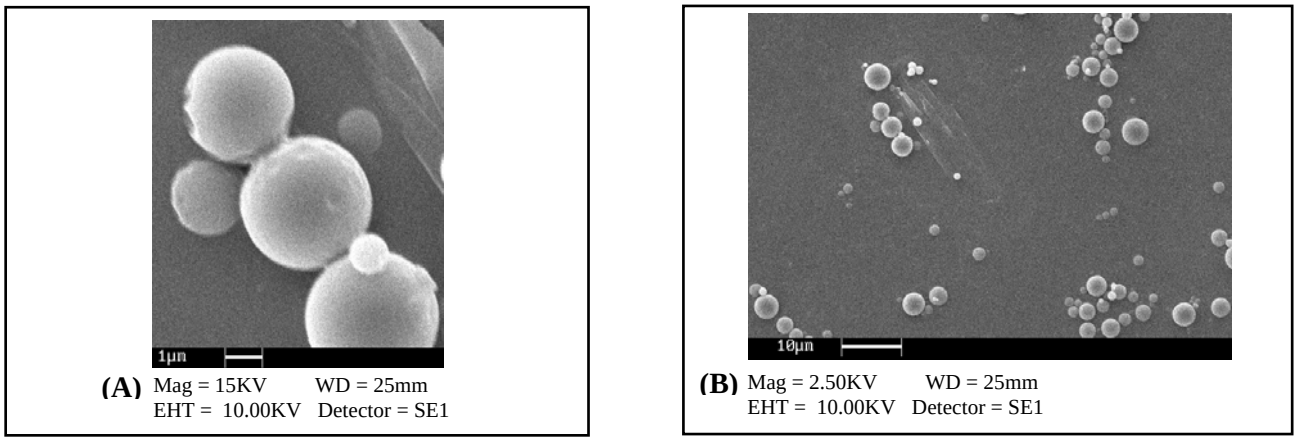

Figure 3. Scanning electronic photomicrographs of Cubebin loaded PLGA microparticles: (A) isolated magnified particle and (B) panoramic view of Cubebin loaded PLGA microparticles.

The method for Cubebin determination on microparticles was developed in view of HPLC parameters used by Bastos J. K. et al., (1996) on Cubebin isolation studies. The mobile phase

initially evaluated was methanol: water. The evaluating of Cubebin using as mobile phase different proportion of methanol: water (70:30, v/v; 60:40, v/v) result in chromatographic peaks with broad bands and different retention time. When the proportion of organic solvent was decreased there was decrease retention time of Cubebin, in according with mechanism of reverse phase. Additionally in relation to selectivity, the broader peak was observed probably in consequence of the more interaction time between Cubebin and the stationary phase (Carr et al., 1996).

However when acetonitrile was used as organic solvent there was a significant increased on resolution, possibly because acetonitrile belong to other group of selectivity. Considering the proprieties that affect the solvents selectivity, as acidity, basicity and the polarity showed in the solvent-selectivity developed by Snyder L. R., et al. (1993), acetonitrila and methanol show distinct selectivity and acetonitrile because are more polar than methanol have more separation force in reverse-phase liquid chromatography (Snyder et al., 1993).

In this way, some proportion of acetonitrile: water were evaluated as mobile phase and the Cubebin good peak, with retention time of 5.0 min was obtained using the mobile phase acetonitrile: water 60:40 (v/v).

The analytical method linearity shows the proportionality between the analyte concentration and the obtained response (Wilson, 1990). The linear equation was y=83,64x-15,97, the linearity range of the method was 1 to 20µg/mL with excellent correlation coefficient (r = 0.9998) and the precision of standard curve was 2.28%.

The quantification limit was 1µg of Cubebin/mL with accuracy of -3.05% and precision less than 1.5%. The coefficient of variation (CV%) for precision and repeatability were below 3% and for accuracy were below 5%, showing the excellent repeatability, intermediate precision and accuracy of developed method of Cubebin quantification.

As demonstrated in the figure 4 the chromatogram obtained for Placebo (inerte PLGA microparticles) showed peak satisfactory separated from the obtained for Cubebin, showing that the compound can be quantified by high performance liquid chromatography (HPLC) using the method and conditions described.

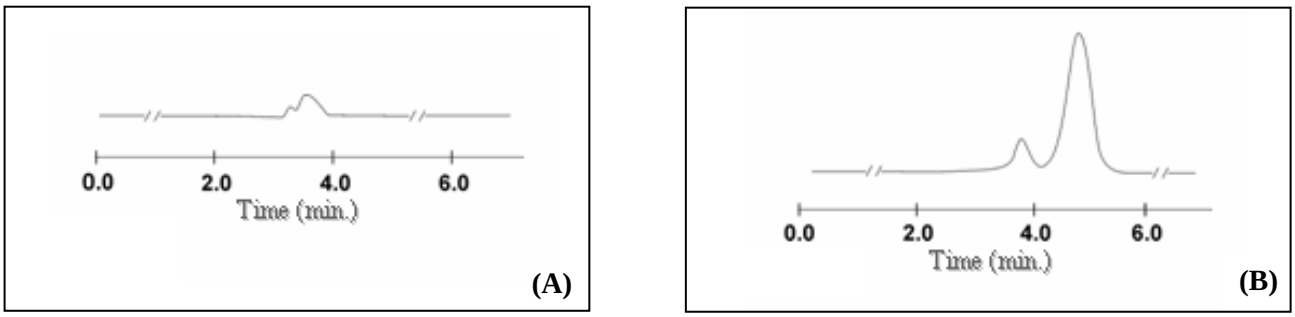

Figure 4. Chromatograms of inert PLGA microparticles sample (A), Cubebin microparticles sample about 6µg of Cubebin/mL (B). Retention time of Cubebin was 5.0min. Chromatographic conditions: C_{18} reversed-phase column shim-pack (250x4mm, particles of 5µm) and a C_{18} pre-column shim-pack (4x4mm, particles of 5µm), mobile phase acetonitrile: water 60:40 (v/v), at flow rate of 1.0mL/min, detection at 285nm.

Conclusions

The preparation methods to be suitable, since the morphological characteristics and efficient yield *in vitro* profile were satisfactory. Thus, the microparticles developed seem to be a promising system

for sustained release of Cubebin. PLGA microparticles can be viable alternative as controlled release device improving the effectiveness and safety of the Cubebin for therapeutically use. The microparticles can promote sustained release, increasing the time intervals between doses and decreasing the discomfort of daily administration. Furthermore, the quantification of compounds biologically actives are essential to determine drugs amount used that guarantee the security and efficacy of utilization and the method developed and used for Cubebin quantification by reversed phase high performance liquid chromatography was adequate for quantification of this compound, showing linearity, precision, accuracy and quantified limit in according with recommended by current pharmaceutical regulatory guidelines. The authors would like to thank CNPq and FAPESP (Brazil) for financial support.

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